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PERFORMANCE CHARACTERISTICS OF ELECTROSPUN CELLULOSE ACETATE NANOFIBER MAT EMBEDDED WITH NANO-ZNO / VITAMINS

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ABSTRACT

Here, we demonstrated two functionalities of cellulose acetate (CA) nanofiber mat, antibacterial property and slow release of vitamins by impregnating nano-ZnO and vitamins (B2 and C), respectively. The morphology of nanofiber mat was studied by SEM and fluorescence microscopy, crystallinity by XRD and chemical nature by FTIR. The nano-ZnO imparted antibacterial activity (>99%) against Staphylococcus aureus (Gram positive) and Escherichia coli (Gram negative). In vitamins loaded CA nanofiber mat, slow release was demonstrated in contrast to burst release in CA films. While slow release of vitamins has potential application in oral delivery system, nano-ZnO impregnation helps to avoid secondary infection.

KEYWORDS: Cellulose Acetate, Oral Delivery System, Electrospinning

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Original Article

INTRODUCTION

Electrospinning is a versatile preparation method for nanofibers from biodegradable and natural polymers for application in tissue engineering and drug delivery.(Rogina, 2014)There are various advantages of nanofibers produced by electrospinning such as high surface area, improved tensile strength, high aspect ratio, flexibility and so on. In addition to the above, electrospun nanofibers have applications in fields such as bone replacement/repair, dental application, wound healing, water / air filtration and nanocomposites. Cellulose acetate (CA) is a versatile polymer for conversion into nanofibers in electrospinning process because of its fiber forming characteristic and highly volatile solvent (acetone). CA has various advantages such as low cost, ready availability and insolubility in water. A detailed review on CA nanofibers(Konwarh, Karak, & Misra, 2013) explored the protocols for preparation and their potential biotechnological applications. The authors have reviewed the loading the fat-soluble vitamins A & E and nano-titania in CA nanofibers for vitamin delivery and photocatalytic activity, respectively.

CA impregnated with zinc oxide nanoparticles (nano-ZnO) exhibit both anti-bacterial and hydrophobic properties.(Anitha, Brabu, John Thiruvadigal, Gopalakrishnan, & Natarajan, 2013) Thus functionalized CA nanofibers have potential applications as antimicrobial surfaces in wound healing, food industry, textiles, packaging and healthcare care products. The well-demonstrated applications of nano-ZnO impregnated electrospun polymeric nanofibers include optoelectronics,(Jiang et al., 2008) photocatalytic degradation of dyes(Liu, Ye, Xiong, & Liu, 2010) and antimicrobial membranes.(Wang, Zhang, Zhang, & Li, 2012)Earlier, PVA electrospun nanofibers were used as fast-dissolving drug delivery system since they possess a highly porousstructure that can disintegrate

instantaneously when placed in the oral cavity, and release the drugs which then dissolve or disperse in the saliva. (Li, Kanjwal, Lin, & Chronakis, 2013) In this work, caffeine and riboflavin were used as the model drugs. Electrospun CA nanofibers act as carriers for delivery of some vitamins to the skin. Certain vitamins which are important for skin healing such as vitamin B2 and vitamin C are delivered to the skin due to its benefits. Vitamin B2 deficiency may cause dry and scaling skin, include cracked and red lips, inflammation of the lining of mouth and tongue, mouth ulcers, cracks at the corners of the mouth (*Angular Chelitis*).Vitamin C plays a key role in collagen formation. The particular connective tissue strengthens the skin, muscles and blood vessels. Studies on human suggest that it can speed healing in many types of wounds. Since both the vitamins (B2 & C) are water soluble, they need to be replace daily in our body system and hence, sustained release mechanism is very much essential during medical treatment for these deficiencies.

While the use of CA nanofibers for release of fat soluble vitamins A & E are studied,(Taepaiboon, Rungsardthong, & Supaphol, 2007) no extensive study is available for release of water soluble vitamins B2 & C. A recent study focused on the use of biocompatible poly-caprolactone nanofibers for loading of drugs along with vitamin B12 for sustained release during transdermal patch applications.(Madhaiyan, Sridhar, Sundarrajan, Venugopal, & Ramakrishna, 2013) In this work, we have demonstrated the two different functionalities of CA nanofibers *viz*., antibacterial property by impregnating nano-ZnO and slow release characteristics of vitamins B2 and C.

EXPERIMENTAL

Materials

Cellulose acetate (white powder; MW = 30 kDa; acetyl content = 39.7% (w/w); degree of acetyl substitution ~2.4) was purchased from Sigma Aldrich (USA). Acetone and DMF were purchased from Fisher Scientific (Mumbai, India), Vitamin B2 from BHD Biochemicals®, England and Vitamin C from Hi-media® (Mumbai, India). Nano-ZnO was prepared in-house and used. For preparation of nano-ZnO, 250 mL of 0.3 M zinc nitrate solution was kept under stirring condition, while adding 250 mL of 3 M sodium hydroxide solution drop-wise using a separating funnel. Appearance of turbidity indicates the progress of reaction and after complete addition of alkali, reaction was allowed to proceed for 1 h. The formed nano-ZnO was purified by repeated centrifugation (first time using water and second time using methanol). The particle size distribution of nano-ZnO was analyzed by DLS analyzer (Nicomp® 360 ZLS) and the absorbance was determined by UV-vis Spectrophotometric analysis.

For electrospinning assembly, a 20-gauge blunt-ended stainless steel needle (outer diameter 0.9 mm) was used as the nozzle. A Gamma® high voltage DC power supply (0-50 kV) was used to generate a high potential across the nozzle and aluminum sheet that was used as a stationary collector electrode. The emitting electrode of positive polarity was attached to the nozzle of the syringe. The syringe was fixed in a microprocessor controlled syringe pump and tilted five degrees from the horizontal to maintain the constant presence of droplet at the tip of nozzle.

Production of CA Nanofibers by Electrospinning

Electrospinning of CA was achieved using binary mixture of 2:1 acetone:dimethylformamide (DMF) as solvent.The concentrations of CA was varied from 12.5 % to 20.0 % (w/w)to optimize the formation of uniform nanofibers. The distance between nozzle and aluminum sheet was adjustedin the range of 15 – 20 cm while the flow rate of polymer solution was kept at an optimized rate of 0.6 mL/h. The entire setup was operated in a fume hood for ventilating solvent vapour.

Preparation &Characterization of Nano-ZnO Embedded CA Nanofibers

Nano-ZnO (50 mM) was added in CA polymer solution and sonicated for an hour to obtain a uniform dispersion. Electrospinning was carried out as given above and the nanofibers obtained were kept at 80 °C overnight to remove the solvent residues. The prepared nano-ZnO incorporated CA fibers were deacetylated in order to remove the acetyl groups from the CA nanofibers. Deacetylation was carried out by aqueous hydrolysis in 0.05 M NaOH solution for 30 h at room temperature and thoroughly rinsed off in distilled water until the pH of nanofiber mat reached 7, followed by drying at 50 °C for 4 h.(Khatri, Wei, Kim, & Kim, 2012) Surface morphology of the nano-ZnO impregnated CA nanofibers was studied using optical microscopy, SEM, crystallinity by XRD (X-ray diffraction) and chemical nature by FTIR (Fourier Transform Infrared Microscope). The zinc content was analyzed using AAS (Atomic Absorption Spectrometry).

XRD analysis was carried out using a Philips PW 1710 X-ray diffractometer with nickel filtered Cu Kα (λ=1.54Å) radiation and analyzed using automatic powder diffraction (APD) software to determine the width angle X-ray diffraction patterns. The diffraction intensities were recorded from 10 to 80 °2θ angles. For FTIR analysis, the sample was analyzed using an IR Prestige21® FTIR with DRS (Diffuse Reflectance Accessory) attachment. The spectra recorded were the average of 100 scans and the contribution of background was accounted for during the analysis.

Evaluation of Antibacterial Properties

For qualitative analysis as per AATCC Test Method 147-2012, samples were pre-sterilized by exposure to UV for 2 h. The electrospun CA nanofibers mat was cut into strips of required size (25 mm × 50 mm) and placed on sterile media plates streaked with overnight broth culture of the test organisms, i.e., *Staphylococcus aureus* and *Escherichia coli*. Observations were recorded as formation of zone of inhibition below / near the fabric. For quantitative analysis as per AATCC Test Method 100-2012, samples were cut to 0.2 g discs and duplicate sets were kept, one for 0 h another for 24 h with 0.2 mL of overnight broth culture (1 x 10^5 CFU/mL) of the above said test organisms. After incubation, the flask contents were shaken with 20 mL of sterile distilled water, serially diluted and plated out by standard plate count method.

Preparation &Characterization of Vitamins Loaded CA Nanofibers

Vitamin loaded CA solutions where prepared by dissolving vitamins B2 or C at the levels of 1% and 5% based on the weight of CA powder, respectively, in the acetone/DMF mixture. Electrospinning was carried out using the same parameters as given above. For comparison, both the neat and the vitamin loaded CA films were prepared by solvent casting technique from 5% w/v CA solution in 2:1 v/v acetone/DMF and the same solution that contained either 1% of vitamin B2 or C. The thickness of both the electrospun CA nanofibers mats and the as cast CA films were in between 20-30 µm. Morphology of CA loaded vitamins was studied by SEM analysis.

Evaluation of Vitamin Release Characteristics

The actual amount of loading of vitamins in the samples was quantified by dissolving them in 3 mL of 2:1 v/v acetone/DMF. To the 0.1mL of above solution, 7.9 mL acetate buffer (pH 5.5) was added for vitamin B2 and sodium oxalate buffer for vitamin C. The absorbance of the prepared solutions was analyzed using spectrophotometer after appropriate dilution (0.1 mL of sample with 2.9 mL of respective buffers). The amount of the vitamins present in the as CA nanofibers and films were calculated using the standard curves.

Due to the limited solubility of both vitamins (B2 and C) in the acetate buffer and phosphate buffer solutions, two types of releasing media were prepared. The first releasing medium was prepared by adding 0.5 vol % of a non-ionic surfactant, polysorbate 80 (Tween 80) to the buffer solutions to help solubilizing vitamins from the loaded samples; and referred to as B/T (acetate buffer) and S/T (sodium oxalate buffer). The other releasing medium was prepared by adding 0.5 vol % of Tween 80 and 10 vol % of methanol in the buffer solution and referred to as B/T/M (acetate buffer) and S/T/M (sodium oxalate buffer). Stability of both vitamins (B2 and C) in the releasing media was evaluated at 37 °C by varying the aging period of each vitamin in the medium. The test solution was prepared by dissolving a known amount of vitamins in the releasing media and their concentration was analyzed over a period of time by spectrophotometric analysis. All the release studies were carried out in triplicates and the average values are reported.

Total immersion method was used to study the cumulative release profiles of vitamins from vitamin loaded electrospun CA nanofibers mat and as cast CA films. The samples were immersed in 20 mL of either of the two releasing medium at 37 °C. At a specified immersion time interval between 0 and 8 h, 1 mL of test medium was withdrawn and an equal amount of the fresh medium was refilled. The amount of vitamins in the sample solution was determined using UV spectrophotometer against the standard curve for each vitamin. Based on these analyses, the cumulative amount of vitamins released from the samples at specified immersion period was calculated. Vitamin B2 was analyzed at 444 nm. For vitamin C, 10% thiourea, 2,4-dinitrophenyl-hydrazine solution and 85% sulphuric acid solutions were added to the sample. Vitamin C reacts with 2,4-dinitrophenylhydrazine and produces an osazone which on treatment with 85% H_2SO_4 forms red colored solution that could be analyzed in spectrophotometer at 521 nm.

RESULTS AND DISCUSSIONS

CA Nanofibers

The CA solutions with concentrations in the range 12.5–20% (w/w) were able to electrospun into nanofibers with diameters ranging between 100 and 1000 nm. But, bead-less nanofibers could be obtained only at the concentration of 18% CA solution at 15 kV and a distance of 15 cm with an optimal flow rate of 0.6 mL/h. At higher concentrations of CA (> 18%), beaded fibers were formed and at lower concentrations (<18%), the nanofibers were not continuous.

Characterization of Nano-ZnO

The formation of zinc hydroxide from the precursors is given in equation 1. Zinc hydroxide gets converted to zinc oxide by dehydration during alcohol washing process (equations 2 and 3). Figure 1(a) depicts the particle size distribution of nano-ZnO as analyzed by DLS particle size analyzer and figure 1(b), UV absorbance by spectrophotometer. The mean diameter nano-ZnO was 24.6 nm ± 3.3 nm and maximum UV absorbance was at 374 nm, corresponding to its band gap.

$$Zn(NO_3)_2 \cdot 4H_2O + NaOH \longrightarrow Zn(OH)_2 + NaNO_3 \quad (1)$$

$$Zn(OH)_2 \longrightarrow Zn^{2+} + 2OH^- \quad (2)$$

$$Zn^{2+} + 2OH^- \longrightarrow ZnO + H_2O \quad (3)$$

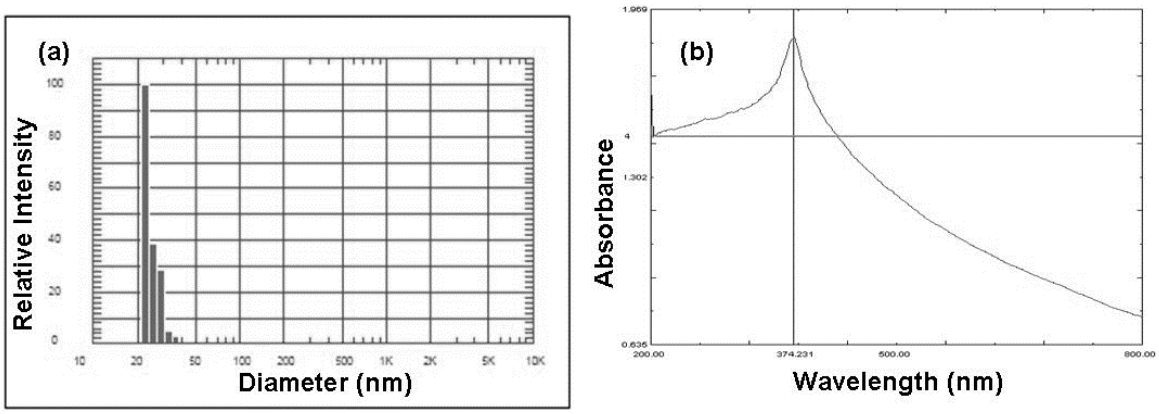

Figure 1: Particle Size Distribution (a) and UV-Absorbance Spectrum (b) of Nano-ZnO

Characterization of Nano-ZnO Impregnated CA Nanofibers

As determined by atomic absorption spectroscopy, out of 50 mM nano-ZnO added in the CA polymer solution, 25 mM got incorporated into the final CA nanofibers. Nano-ZnO impregnated CA nanofibers were produced in the same way as that of CA nanofibers without changing any parameters. Deacetylation of CA nanofibers as well as nano-ZnO impregnated CA nanofibers was carried out by treating with NaOH that hydrolyses the acetate group. CA nanofibers do not fluoresce under the fluorescence microscope while the nano-ZnO has the fluorescence ability due to its band gap (corresponding to the absorbance in the UV region at 374 nm). Hence, the nano-ZnO impregnated CA nanofibers showed spotted fluorescence all through the nanofibers indicating the uniform distribution of the nano-ZnO particles. Figure 2 shows the fluorescence micrographs of nano-ZnO impregnated CA nanofibers (with and without green filter).

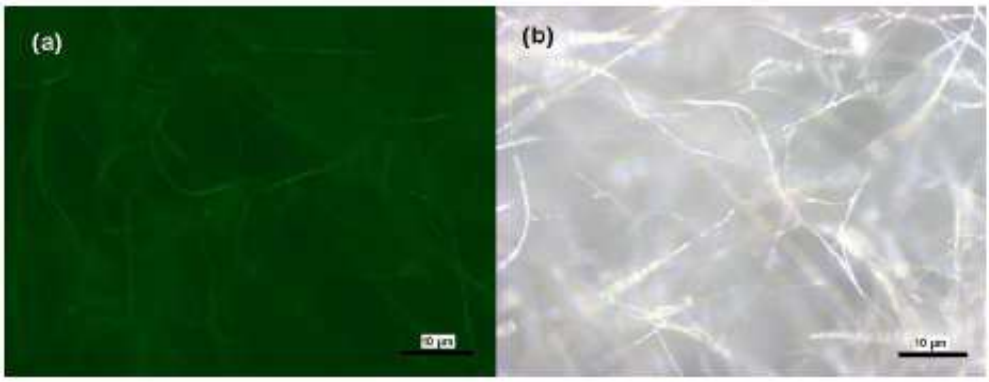

Figure 2: Fluorescence Micrograph of Nano-ZnO Impregnated CA nanofibers with (a) and without (b) Green Filter

It is well known that the morphology of the electrospun fibers are strongly dependent on the processing parameters such as polymer concentration, voltage and distance between the electrodes. The polymer concentrations in the solution have an influence on the morphology of fibers which were examined by SEM. At lower concentrations, beads are formed along the fiber that is generally considered as defects. These beads may decrease the effective surface area of the fibers. The number of beads formed is reduced as we go from 6 to 15 wt% and the beads completely disappeared in the case of 18 wt% of the solution. However, there is a considerable increase in the fiber diameter. The average diameter of the optimized fiber was found to be 670 ± 60 nm as derived from SEM micrographs given in figure 3. The increased polymer concentration in the solution helps to prepare the uniform defect free fiber because at higher polymer concentrations, sufficient chain entanglements serve to stabilize the jet by inhibiting its breakup. CA nanofibers, before and after

deacetylation showed uniform web structure without any significant difference since deacetylation only affects the surface chemistry.

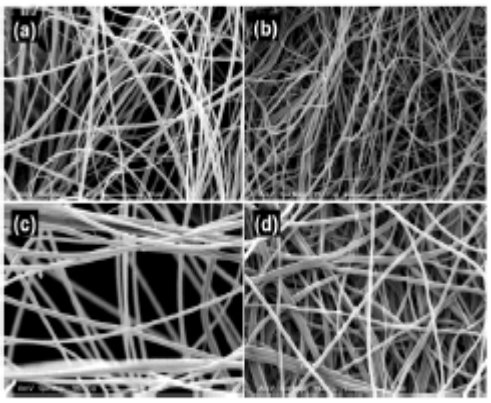

Figure 3: Scanning Electron Micrographs of CA Nanofibers (a), Deacetylated CA Nanofibers (b), Nano-ZnO Impregnated CA Nanofibers (c) and Nano-ZnO Impregnated Deacetylated CA Nanofibers (d)

The XRD pattern of CA nanofibers and nano-ZnO impregnated CA nanofibers are given in figure 4. The CA nanofibers (both native & deacetylated) exhibited the diffuse characteristic pattern of an amorphous phase. This corroborates well with the reported literature value.(Weitao, Jianxin, Shizhong, & Weidong, 2011) In case of nano-ZnO impregnated CA nanofibers (both native & deacetylated), peaks were observed in between 30–40 °2θ corresponding to (100), (002) and (101) faces that could be indexed as the hexagonal *wurtzite* structure of ZnO(Prasad, D'Souza, Yadav, Shaikh, & Vigneshwaran, 2006) and they match well with standard diffraction data (JCPDS: 36145).

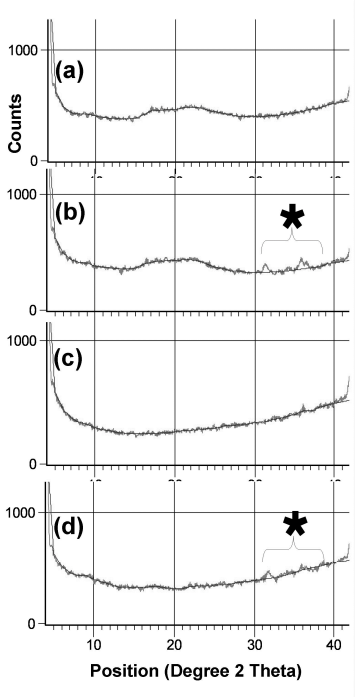

Figure 4: X-ray Diffraction Patterns of CA Nanofibers (a), nano-ZnO impregnated CA nanofibers (b), Deacetylated CA Nanofibers (c) and Nano-ZnO Impregnated Deacetylated CA Nanofibers (d). Star Marks Indicate the Peaks Correspond to the Crystalline Planes of Nano-ZnO

Figure 5 shows the FTIR analyses of CA nanofibers before and after deacetylation. The main characteristics bands of CA were assigned as follows: The absorption peak at 3500 cm^{-1} can be attributed the presence of hydroxyl group and the peaks at 1743, 1250 and 1050 cm^{-1} correspond to stretching of C–O group, ether group and –C–O– bond of the $–CH_2–OH$ group, respectively. While comparing the control and deacetylated samples, the peak at 1250cm^{-1}(corresponds to -C-O- stretch)(Tosh, 2011) was not observed in deacetylated sample indicating the removal of acetyl group during deacetylation process. The deacetylation process is primarily carried out to reduce the solubility of nanofibers, thereby making it stable for further use.

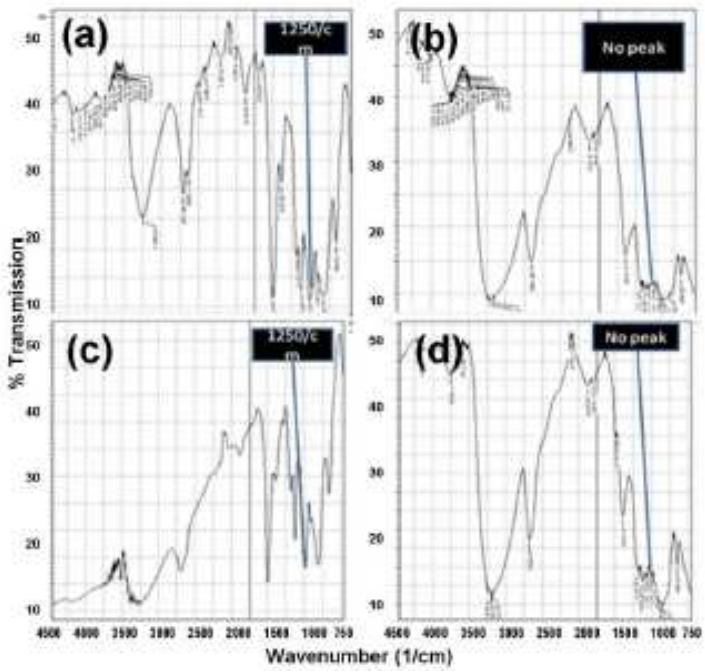

Figure 5: FTIR Spectra of CA Nanofibers (before 'a' and after 'b' Deacetylation) and Nano-ZnO Impregnated CA Nanofibers (before 'c' and after'd' Deacetylation)

Antibacterial Property of Nano-ZnO Impregnated CA Nanofibers

The antibacterial property of CA nanofibers and nano-ZnO impregnated CA nanofibers (both native and deacetylated) were evaluated against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria. While neat CA nanofibers did not show any inhibition, nano-ZnO impregnated CA nanofibers showed significant inhibition zone around the samples. It is seen that the nano-ZnO had a stronger influence on *S. aureus* (4 cm diameter) than *E. coli* (3 cm diameter) as per the zone of inhibition. The nature of cell wall structure is one of the possible reasons for observed difference in sensitivity. The *S. aureus* is composed of multi layers of peptidoglycan which has plenty of pores that could render them more susceptible to the intracellular transduction of nano-ZnO. In contrast, the cell wall of *E. coli* is relatively thin mainly consisting of peptidoglycan and an outer layer of lipopolysaccharide, lipoprotein, and phospholipids, which would be less prone to the attack of the nanoparticles. Therefore, the nano-ZnO had 50% higher antibacterial activity against *S. aureus* than *E. coli.* Antibacterial activity of nano-ZnO impregnated CA nanofibers and its deacetylated samples studied by quantitative methodology showed 99% reduction for both *S. aureus* and *E. coli.* Hence, nano-ZnO at a concentration of 2.5 mM in CA nanofibers can cause complete bacterial inhibition.

Vitamins Loaded CA Nanofibers

Neat and vitamin B2 & C loaded CA nanofibers were prepared keeping the optimized electrospinning parameters. Simultaneously, CA films were prepared by solvent casting process for comparison.The actual amount of the vitamins incorporated in the vitamins loaded electrospun CA fiber mats and CA films were determined prior to investigation on release characteristics. The actual amount of vitamin B2 that got incorporated into CA nanofibers & films were determined to be 23.3% and 45.9% respectively. On the other hand, the actual amount of vitamin C incorporated into CA nanofibers & films were 41.9% and 72.7%, respectively. The variation in percentage is contributed by differential solubility of vitamin B2 & C and differences due to process conditions (electrospinning and film casting). Prior to the release studies, the stability of the vitamins in the releasing media was investigated. Among the two media, B/T/M and S/T/M, vitamin B2 was stable in both while vitamin C got oxidized to dehydroascorbic acid (reversible reaction) and subsequently to 2,3-diketo -L-gulonic acid which is an irreversible reaction. Hence, a detection method was selected in which both ascorbic as well as dehydroascorbic acid are detected. Even then, the stability of vitamin C was not significant in the media but, it was very much stable after incorporation into the CA nanofibers and / or films.

The release characteristics of vitamins loaded CA nanofiber mats and films were carried out by total immersion method.[7] The experiments were carried in B/T and B/T/M releasing media at the physiological temperature of 37°C for vitamin B2 & C, respectively. The cumulative amount of the vitamins released (reported as the percentage of the actual amount of vitamins present in the samples) is given in Figure 6. The Figure 6(a) and 6(b) represent the release characteristics of vitamin B2 in B/T and B/T/M media, respectively; while Figure 6(c) and 6(d) represent that of vitamin C in B/T and B/T/M media, respectively. In these figures, the blue line (with diamond dots) represents the data for CA nanofibers and the red line (with square dots) represents that of CA films. The overall release behaviour of vitamin B2 in both the media are not significant (<35%) as compared to that of vitamin C during the 8 h of study duration. In B/T medium, only 18% of vitamin B2 was released by film while 25% by nanofibers after 8 h. In case of B/T/M medium, only 19% of vitamin B2 was released by film while 32% by nanofibers & still showing the increasing trend. In spite of films holding more amounts of vitamins than that of nanofibers, the release by nanofibers is better. In case of vitamin C, there were burst release from films both in cases of B/T and B/T/M media to the extent of 80% and 95%, respectively. In nanofibers, a steady release of vitamin C was noticed to the extent of 35% and 95%, respectively for B/T and B/T/M media for the total period of 8 h. This demonstrates the potential of CA nanofibers to control release the vitamins over a period of time.

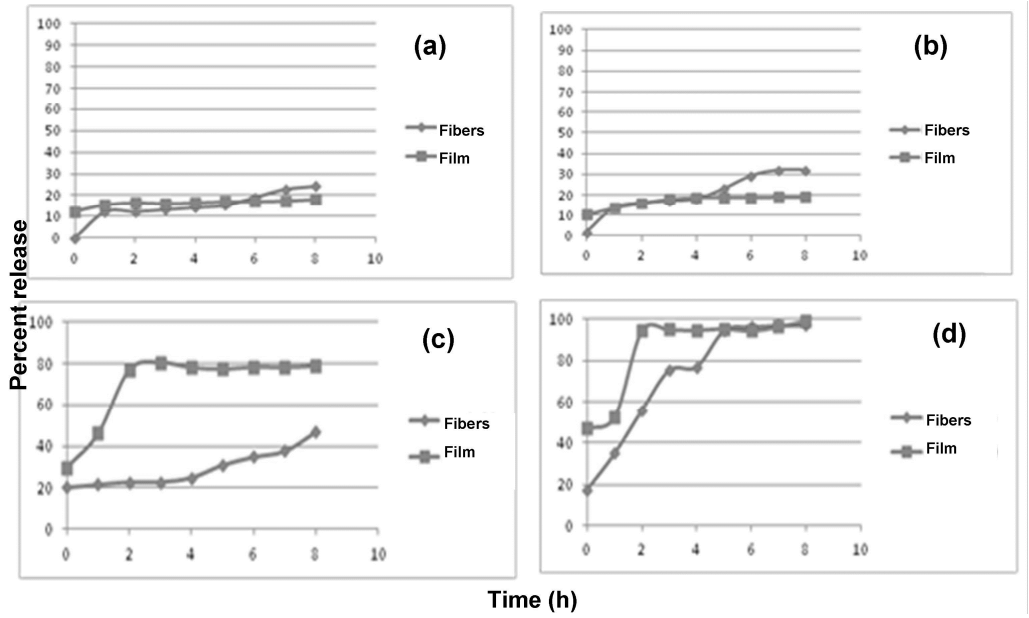

Figure 6: Release Characteristics of Vitamin B2 in B/T (a) and B/T/M (b) Media and Vitamin C in B/T (c) and B/T/M (d) Media

Figure 7 shows the SEM micrographs of vitamin loaded films / nanofibers before (Figures 7a to 7d) and after (Figures 7e to 7h) release study. While significant difference was observed in the surface morphology of films before and after release of vitamins, no such difference could be noticed in nanofibers. The reasons could be attributed to the heavy loading of vitamins (and corresponding release) in films compared to that of nanofibers. Recently, an attempt was made to load the vitamin B12 along with drugs into hydrophobic polymeric nanofibers for sustained release in transdermal patch applications.(Madhaiyan et al., 2013)Another work reported the use of chitosan in polycaprolactone nanofiber matrix that avoided the initial burst release and also reduced *Staphylococcus aureus* growth; and, such bioactive bandages may serve as versatile and less expensive alternatives for the treatment of complex wounds.(Guadalupe et al., 2015)In line with this, our findings will help to diversify the use of CA nanofibers for sustained release of vitamins B2 and C when used in oral delivery system. In addition, nano-ZnO could be incorporated into the CA nanofibers system to avoid secondary infections.

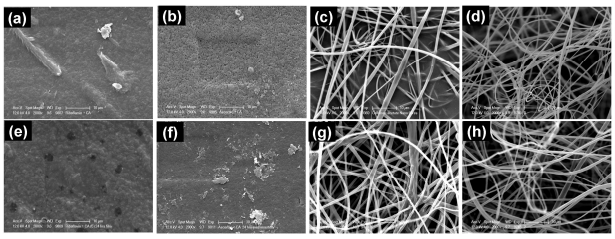

Figure 7: Scanning Electron Micrographs of Vitamin B2 Loaded Film (before '7a' and after '7e' Release), Vitamin C Loaded Film (before '7b' and after '7f' release), Vitamin B2 Loaded Nanofibers (before '7c' and after '7g' Release) and Vitamin C loaded Nanofibers (before '7d' and after '7h' Release)

CONCLUSIONS

Two different functionalities (antibacterial property and slow release of vitamins) were introduced into the nanofiber mat of cellulose acetate and their properties were evaluated. The stability of the cellulose acetate nanofibers impregnated with nano-ZnO was improved by partial deacetylation process that rendered itself insoluble in water. The nano-ZnO imparted antibacterial activity (>99% against both *S. aureus* and *E. coli)* to the cellulose acetate nanofibers.

Also, the slow release of vitamins (B2 and C) loaded in CA nanofibers was demonstrated in the defined media in contrast to burst release in case of CA films. While the slow release of vitamins help to develop oral delivery system, nano-ZnO impregnation helps to avoid the secondary infection.

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